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Genetics

Transmission electron microscopic method for gene mapping on polytene chromosomes by *in situ* hybridization

(colloidal gold spheres/A·T base pairing/photochemical crosslinking)

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ABSTRACT **A transmission electron microscope method for gene mapping by *in situ* hybridization to *Drosophila* polytene chromosomes has been developed. As electron-opaque labels, we use colloidal gold spheres having a diameter of 25 nm. The spheres are coated with a layer of protein to which *Escherichia coli* single-stranded DNA is photochemically crosslinked. Poly(dT) tails are added to the 3′ OH ends of these DNA strands, and poly(dA) tails are added to the 3′ OH ends of a fragmented cloned *Drosophila* DNA. These probe–dA strands are hybridized *in situ* to polytene chromosome squashes. Gold spheres are linked to the hybridized probe–dA strands by A·T base pairing. The sphere positions relative to the chromosome bands can be observed by transmission electron microscopy. The method shows low background and high resolution.**

In situ hybridization of ^{3}H- or ^{125}I-labeled nucleic acids to their complementary sequences on polytene chromosomes is an effective direct physical method for mapping cloned genetic sequences on chromosomes (1). Recent developments suggest that this method can be extended to mapping cloned sequences on mammalian metaphase chromosomes (2–5). The resolution of the method is limited by the size of the silver grains developed, and the sensitivity is limited by the specific activity of the available suitable radioactive labels.

We wished to develop a transmission electron microscopic method for gene mapping by *in situ* hybridization onto chromosomes. Such a method has the potential for providing higher resolution and greater sensitivity. We chose to use colloidal gold spheres having diameters of ≈25 nm as the electron-opaque label to attach to the specific nucleic acid probes, which were hybridized to their complementary sequences in the chromosomes.

The general strategy for the technique is depicted in Fig. 1. A gold sol with a predetermined uniform size was prepared by reduction of acidic gold chloride with citric acid (6). Bovine serum albumin (Alb), like many other proteins, will adsorb on the surface of the gold particles, thus decreasing their tendency to aggregate. Addition of polyethylene glycol to the solution greatly increases the stability of the sol (7).

To crosslink DNA to the Alb-coated gold particles, nitrene groups that can be photoactivated were covalently attached to the amino groups of the Alb by amide bonds. At pH 4 and low electrolyte concentration, this modified protein shell of the gold particles is positively charged and will bind single-stranded DNA electrostatically. By photolysis of the nitrene groups, the DNA was covalently linked to the protein coat of the spheres. We observed that the multiple DNA crosslinks greatly increased the stability of the sol. Poly(dT) tails were added to the single strands of the DNA by terminal deoxynucleotidyltransferase (terminal transferase)-catalyzed addition of thymidine residues. Similarly, poly(dA) tails were added to exposed 3′ OH ends of a cloned DNA containing a *Drosophila* sequence.

The poly(dA)-tailed cloned DNA was hybridized to a squash of *Drosophila* polytene chromosomes on a glass slide. On incubation, the gold/poly(dT) binds by A·T base pairing to the chromosomal sites at which the dA-tailed probe DNA has hybridized. The squash was then transferred to an electron microscope grid. By careful correlation of the images of the squash in the light microscope and in the electron microscope, the positions of the bands can be identified in the latter. The chromosomal location of the sequences in the cloned DNA can then be mapped by identifying the region containing the gold markers in the electron micrographs.

MATERIALS AND METHODS

Preparation of Gold Spheres Crosslinked to DNA. Gold sols with particles 25 ± 5 nm in diameter were prepared according to Frens (6). Sodium citrate (1%, 0.2 ml) was added to 30 ml of boiling double-distilled H_2O and then 0.3 ml of 1% $HAuCl_4$ was gradually added with constant stirring. After ≈2 min, the color rapidly changed to a brilliant red, indicating the formation of a monodisperse colloidal solution. The solution was refluxed for 30 min, and 0.3 ml of bovine serum ovalbumin (5 mg/ml; Pentex) and 1.5 ml of 1% polyethylene glycol (Carbowax 20-M, Union Carbide) were added with gentle stirring. After an additional 30 min, the solution was centrifuged in an SW41 rotor at 4°C for 20 min at 25,000 rpm. The pellet was suspended in 3 ml of CPS buffer [Carbowax 20-M (0.1 mg/ml)/0.01 M sodium phosphate, pH 6.8/0.15 M NaCl]. The gold/Alb sol can be stored in this buffer for several months at 4°C. This solution (0.2 ml) was layered onto 0.5 ml of 20% sucrose in CPS buffer in each of four plastic Eppendorf centrifuge tubes and centrifuged at 4°C for 12 min. The resulting pellets were suspended in 200 μl of 5 mM phosphate buffer, pH 7.2/Carbowax, (0.05 mg/ml) and transferred to small glass test tubes. All subsequent steps were carried out in the dark until the photolysis reaction. The stock solution of nitrene was *N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate (4 mg/ml; Pierce). It can be stored in the dark at 20°C for up to 2 weeks. Twenty microliters of this solution was mixed with the gold/Alb solution. After incubation for ≈12 hr, 3 μl of 1 M glycine was added to remove any excess succinimidyl ester.

For test reactions to develop the crosslinking procedure, we used single-stranded ^{3}H-labeled *Escherichia coli* DNA sheared

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Abbreviations: Alb, bovine serum albumin; kb, kilobase(s); NaCl/Cit, standard saline citrate; gold/dT, gold/DNA/poly(dT) spheres.

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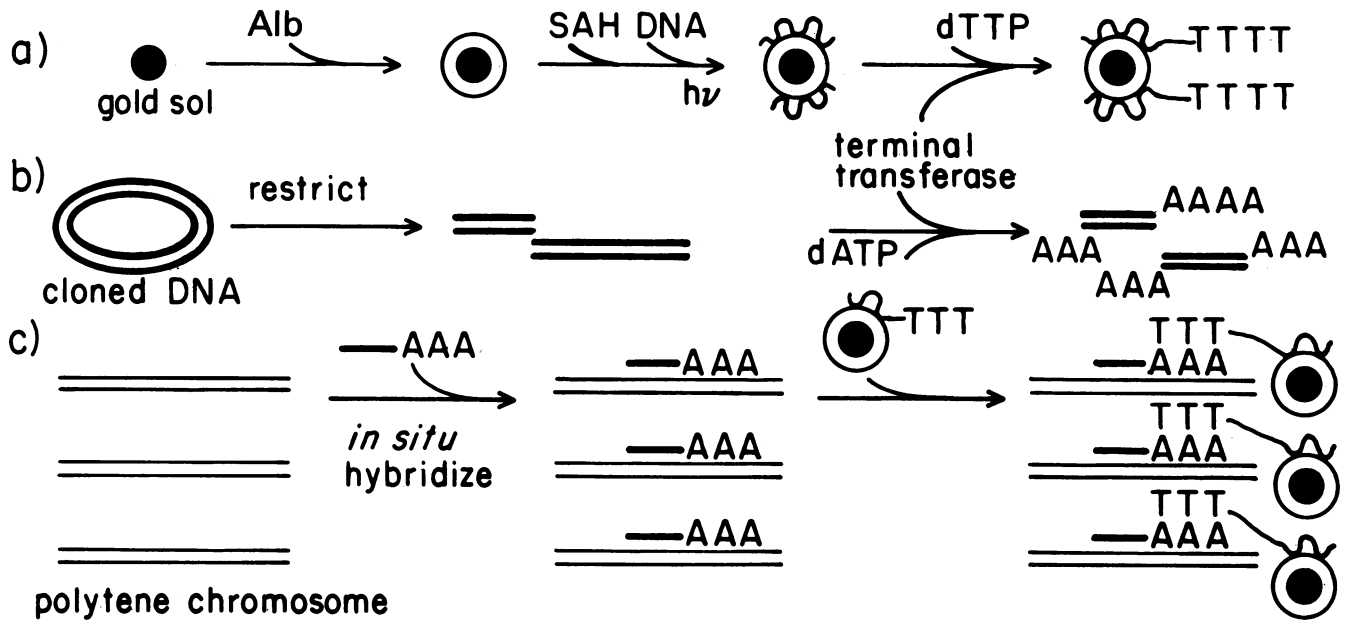

FIG. 1. Procedure for preparing gold labels for electron microscopic gene mapping. SAH, *N*-succinimidyl-6-(4'-azido-2' nitrophenylamino) hexanoate.

to an average length of 1.8 kilobases (kb) in a Virtis apparatus. For making the gold/DNA/poly(dT) labels for *in situ* hybridization, we digested unlabeled *E. coli* DNA with *Eco*RI and then sonicated the digests to an average length of 0.4 kb. Fifty microliters of the single-stranded DNA (1.2 mg/ml) was added to the nitrene-modified gold/Alb solution 10 min after adding the glycine. After 30 min, 3 μl of 1 M HCl was added to adjust the pH to 3 to 4. The solution was placed in a quartz cuvette and exposed for 10 min with constant magnetic stirring to radiation from a long-wave-length medium-pressure 85-W mercury lamp (General Electric HB 85) 12 cm away. The gold spheres were sedimentated through a 10–25% sucrose gradient in CPS buffer at 30,000 rpm for 30 min in an SW41 rotor to remove excess uncoupled DNA. The resulting pellet of gold/Alb/DNA (gold-DNA) was suspended in 100 μl of CPSE buffer (CPS buffer/5 mM Na_3 EDTA). The amount of DNA bound to the gold was estimated by measurement of the amount of cosedimented [^{3}H]DNA label and also by electron microscopic observation of formamide–cytochrome *c* spreads of the gold/DNA solution (8). Gold/DNA can be stored in CPSE buffer at −20°C for up to 1 month.

Terminal Transferase Reactions. These reactions were carried out essentially as described (9). TT buffer consisted of 140 mM cacodylic acid/30 mM Tris·base adjusted to pH 6.9 with KOH/0.1 mM dithiothreitol/1 mM $CoCl_2$. Two hundred microliters of gold/DNA solution was pelleted through a 20% sucrose block in TT buffer twice in an Eppendorf centrifuge and then suspended in 50 μl of TT buffer/3 mM [^{3}H]dTTP. Thirty units of terminal transferase was added, and the solution was incubated at 37°C for 1 to 2 hr. The resulting gold/DNA/poly(dT) (gold/dT) spheres were sedimented through a sucrose gradient and suspended in 50 μl of CPSE buffer. Radioactivity that cosedimented with the gold was measured.

To prepare probe–dA, 10 μg of double-stranded probe DNA was digested with restriction endonuclease, ethanol precipitated, and suspended in 50 μl of TT buffer/3.2 mM dATP containing 30 units of terminal transferase. After incubation at 37°C for 1.5 hr, the reaction mixture was loaded onto a 0.4 ml hydroxylapatite column that had been equilibrated with 0.01 M phosphate, pH 7.2. The column was washed with 0.04 M and 0.12 M phosphate to remove free nucleotides and single-stranded self-polymerized poly(dA), and the tailed double-stranded cleavage fragments were eluted with 0.4 M phosphate. The addition of poly(dA) tails to the cleavage fragments was assayed by gel electrophoresis and electron microscopic observation. The double-stranded probe–dA was denatured in 0.1 M NaOH for 2 min, neutralized with Tris·HCl, and diluted with loading buffer (0.5 M NaCl/1 mM EDTA/10 mM Tris, pH 7.5/1% Na *N*-lauroylsarcosine. It was then loaded on a previously washed oligo(dT)-cellulose column equilibrated with loading buffer. After extensive washing with loading buffer, dA-tailed DNA was eluted with 5 mM Tris/5 mM EDTA, pH 7.5/0.05% Na *N*-lauroylsarcosine, concentrated by ethanol precipitation, and suspended in 0.30 M NaCl/0.03 M Na citrate (2× NaCl/Cit) base 10 mM Tris, pH 8.2/10 mM Na_3 EDTA/50% formamide just before use for *in situ* hybridization.

Preparation of Chromosomes and *in situ* Hybridization. The procedures for the preparation of chromosomal squashes and *in situ* hybridization are essentially as described by Gall and Pardue (1). The slides were treated with RNase A (1 μg/ml) in 2× NaCl/Cit at 37°C for 1 hr, washed twice in 2× NaCl/Cit/5 mM Tris, pH 7.5/5 mM EDTA at room temperature for 4 hr, rinsed in 70% and 90% ethanol, and air dried. Hybridization was carried out using probe–dA at 10 μg/ml in 2× NaCl/Cit/10 mM Tris, pH 8.2/10 mM Na_3EDTA/50% formamide for 17 hr at 37°C. The slides were then washed four times in 2× NaCl/Cit/5 mM Tris, pH 8.2/10 mM Na_3EDTA at room temperature for a total period of 2 hr, rinsed in CPSE buffer and incubated with 20–40 μl of gold/DNA/dT in CPSE buffer at room temperature for 2 hr. Slides were washed four times with CPSE buffer at room temperature for a total period of 1 hr, briefly rinsed with H_2O, air dried, dipped in 0.7% parlodion in amyl acetate, air dried, and examined by phase-contrast microscopy to select suitable areas with good chromosome spreads. A razor blade was used to cut out several suitable areas and a 100-mesh copper grid was placed over the selected chromosome squashes under a phase-contrast microscope. The surface parlodion film with the grid was carefully floated off on a

surface of 0.3 M HF. Grids were then picked up on a small drum made of Saran Wrap over a small beaker, gently rinsed with H_2O, reexamined by phase-contrast microscopy to make sure that the chromosome squashes had been properly transferred, and then examined by transmission electron microscopy at 80 keV with a Philips 300 or a JEOL CX 100 instrument.

RESULTS

Preparation and Properties of Gold Spheres Coated with Protein and Then with DNA. The suspension of 25-nm particles has a maximum light absorbance at 525 nm. This absorbance was used to monitor the concentration of gold particles during the preparation. The photochemical crosslinking agent [*N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate] dissolved in dioxane, has an absorption peak at 465 nm. On photolysis, the peak shifts to 380 nm. This property was used to determine the necessary time of irradiation for complete photolysis.

Efficient crosslinking of the hexanoate-modified gold/Alb particles to DNA was achieved by photolysis of the mixture at a low electrolyte concentration and a pH at which the spheres were positive and the DNA was negative so that these two components were electrostatically bound to each other. In one set of experiments, single-stranded [^{3}H]DNA sheared to an average length of 1.8 kb was used. An electron micrograph of a cytochrome *c* spread of gold/Alb is shown in Fig. 2*a* and one of the gold/DNA is shown in Fig. 2*b*. From contour-length measurements of all the DNA filaments emanating from a single particle, we estimate that there are $\approx 2 \times 10^4$ nucleotides of DNA per sphere or $\approx$10 DNA strands. Measurements of the radioactivity pelleting with the sphere give concordant values within experimental error. About 10% of the input DNA is bound to the spheres and the recovery of the spheres in the procedure is 30–40%.

Fig. 2*d* Inset shows a high-magnification micrograph of gold/Alb in a cytochrome film after Pt/Pd shadowing. The layer of Alb around the gold is readily observed. As shown in Fig. 2*a*, the protein-coated gold spheres do not stick to purified DNA under conditions used for preparing a basic protein film for electron microscopy. However, these spheres are not suitable for *in situ* hybridization because they have a relatively high affinity for glass slides and for various cellular components. Sometimes they form large aggregates during manipulation or during incubation of chromosome preparations. The spheres become much more stable after crosslinking with DNA. There is less tendency to aggregate, less tendency to adhere to glass surfaces, and very low nonspecific sticking to chromosomes.

Addition of Poly(dT) and Poly(dA) Tails. We have used DNA cleaved by shear or restriction endonuclease digestion to produce a suitable substrate for addition of poly(dT) or poly(dA) tails by the action of terminal transferase in the presence of the appropriate nucleoside triphosphate. Sonicated DNA probably contains free 3′ OH ends and thus is a suitable substrate for the terminal transferase reaction (10). For the addition of poly(dA) tails to restriction endonuclease-cleaved probe DNA, the qualitative fact that significant addition has occurred can be demonstrated by gel electrophoresis. It is also possible to observe the single-stranded tails on the duplex DNA and to measure their length by electron microscopy (Fig. 2*c*). These micrographs show tails at the ends of the double-stranded DNA and in some instances at nicks within the duplex. The average length of the tails is $\approx$0.5 kb.

It is difficult to determine the number and length of the poly(dT) tails added to the gold/DNA particles. In a test experiment, the 8.3-kb plasmid pCIT19 was linearized by digestion with *Pst* I and tailed. The poly(dT) tails on the duplex DNA had an average length of 0.8 ± 0.1 kb. Free poly(dT) filaments, possibly formed on contaminating low molecular weight primers, were also observed and had a length of $\approx 0.8 \pm 0.2$ kb. When comparable reactions were carried out with gold/DNA preparations containing unlabeled *E. coli* DNA 0.4 kb long, free strands of poly(dT) 0.8 ± 0.1 kb long were observed in the background. By assuming the same length for poly(dT) attached to gold/DNA, from the total amount of radioactivity due to incorporated [^{3}H]thymidine that cosedimented with the gold after the terminal transferase reaction, we estimated that various preparations had 7–15 poly(dT) tails per gold particle.

Fig. 2*d* shows the results of an experiment in which a nicked circular plasmid having, on average, 1.8 poly(dA) tails per molecule of average length 0.46 kb was incubated with poly(dT)-tailed gold spheres prepared as described above. It can be seen that all of the plasmid molecules have several gold spheres attached. Thus, we observe that the basic scheme for electron microscopic labeling of DNA molecules containing poly(dA) tails by spheres containing segments of poly(dT) works.

Labeling of Probe Complementary Sequences in Polytene Chromosomes. By using the procedure described for mounting chromosomes, it is possible to correlate band structure as ob-

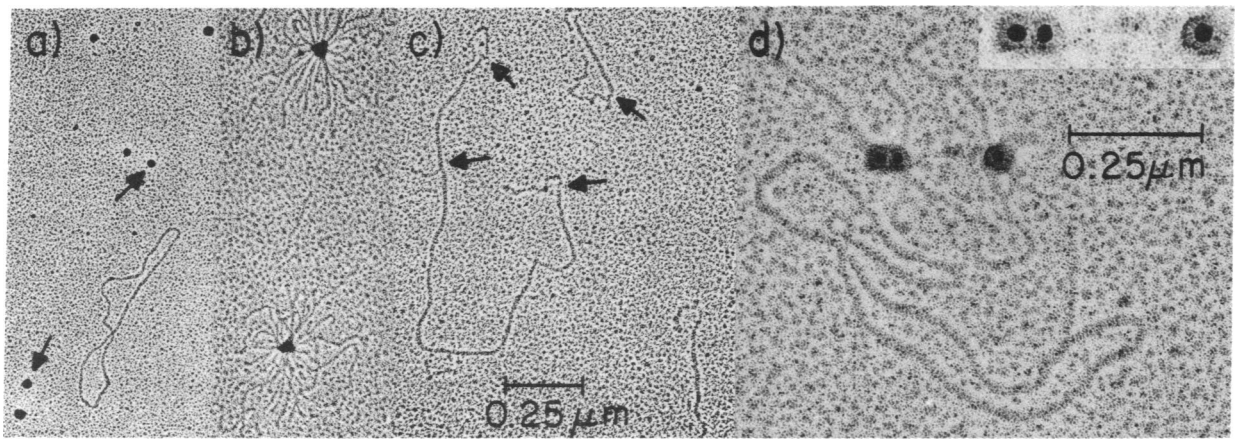

FIG. 2. Formamide/cytochrome *c* spreads. (*a*) Alb-coated gold spheres/ simian virus 40 DNA. (*b*) Gold/Alb crosslinked to single-stranded DNA. (*c*) *Pst* I fragments of pCIT19 with poly(dA) tails. Arrows indicate junctions between double-stranded DNA and poly(dA) tails. (*d*) Product of reaction of circular pCIT19–dA and gold-dT. (*Inset*) Enlarged underexposed photograph of the spheres in *d*. The layer of Alb surrounding the gold can be clearly seen.

served by light microscopy with the structures seen in the electron microscope and thus identify particular bands for further study. Under optimal conditions with native chromosomes, a great deal of detailed structure is observed (Fig. 3). During the alkaline denaturation step in the *in situ* hybridization procedure, much of this fine structure is lost. However, one can still readily identify the banding pattern by correlation of light and electron microscope images.

As a simple meaningful test of this procedure, we have mapped the *Drosophila* 5S rRNA genes, which are known by conventional *in situ* hybridization to map at the 56 EF region (11). The probe used was the plasmid pCIT19, which contains a 1.64-kb DNA insert consisting of three *Drosophila* 5S rRNA genes in a tandem repeat (12). pCIT19 was digested with *Pst* I, which cuts the vector ColE1 twice and does not cut within the insert. In a control experiment, a chromosome squash was hybridized with single strands of pCIT19 that had not been tailed with poly(dA), and the hybrid was then incubated with gold/dT. We did not detect any gold/dT in the 56 EF region nor in any other region. This result shows that gold/dT has a very low background of binding to *Drosophila* polytene chromosomes.

Poly(dA) tails 0.72 kb long were added to the pCIT19 cleavage fragments. The purified denatured DNA was hybridized *in situ* to chromosome squashes. This experiment has been repeated three times using several different batches of gold/dT. At least four 56 EF regions have been studied in each experiment. In each case, clusters of gold sol were observed at 56 EF (Fig. 4*b*), while other nearby puffs did not contain any significant amount of label. The gold spheres were distributed over a region extending from the dense band at 56F toward the less dense 56E region, with heavier labeling in the former. An accurate quantitation of the amount of bound gold spheres is not easy because they are not all located at the same focal level. We did observe, however, that a batch of gold/dT with an estimated 7 poly(dT) tails per sphere gave a higher level of labeling than did a batch with an estimated 15 tails per sphere.

The ≈165 (13) clustered 5S rRNA genes of *Drosophila* have a repeat length of 0.38 kb (12). They probably occur as a single continuous cluster (14), which therefore extends over a length of ≈63 kb on the chromosomal DNA. Although accurate measurements are not possible, the gold spheres defining the positions of the 5S genes (Fig. 4*b*) appear to extend over a region 0.9–1.0 μm long.

To investigate the resolution of the present technique, we have mapped two closely single-spaced copy genes. Each of the recombinant DNA molecules cDm2848 and cDm2188 contains a 16-kb insert of *Drosophila* single-copy DNA in the vector

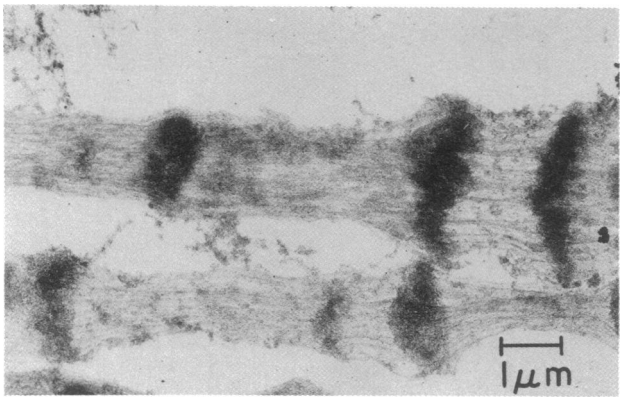

FIG. 3. Transmission electron microscope image of a portion of a *Drosophila* polytene chromosome.

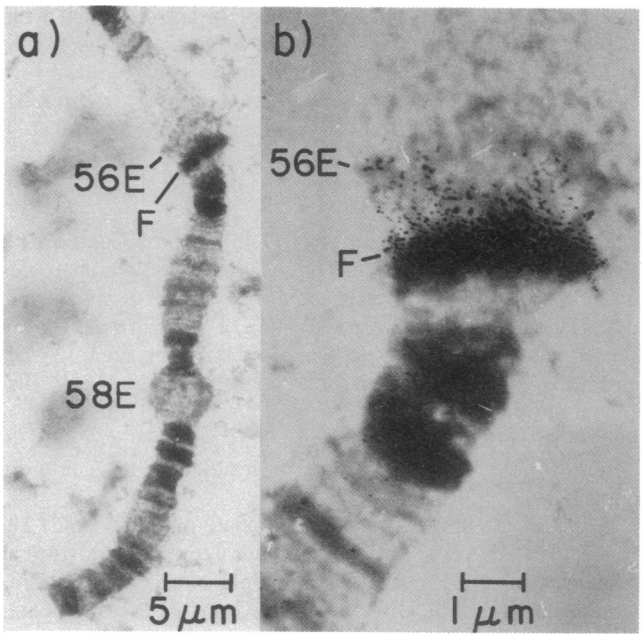

FIG. 4. (*a*) Transmission electron microscope image of part of chromosome 2R hybridized to pCIT19–poly(dA)/gold-(dT) at low (×600) magnification. (*b*) The same chromosome at a higher (×7100) magnification, showing a cluster of gold spheres at the 56 EF region.

Charon 4. These inserts are separated by 263 kb and map at regions 87 D11 and 87E5, respectively (W. Bender, personal communication). Poly(dA) tails were added to the *Eco*RI-digested restriction fragments of these DNAs. When either one of the cloned DNAs was used for *in situ* hybridization, one narrow gold-sphere band was detected in the correct region. When both cloned DNAs were used, two very narrow gold-sphere bands were observed. These bands are 0.9 μm apart in the particular squash shown in Fig. 5*b*. The widths of these narrow bands are difficult to measure but are ≈50 nm.

DISCUSSION

Because of their high electron density and relatively uniform diameters, gold spheres in the size range used here are excellent electron-opaque labels for transmission electron microscopy. Bender and Davidson (15) developed a method for electron microscopic mapping of poly(A) stretches on nucleic acids by hybridization to poly(dT) segments attached to a circular DNA. We have adapted this method to the present problem of labeling cloned DNA hybridized to *Drosophila* polytene chromosomes. To attach poly(dT) tails to the protein-coated gold spheres, we developed a method to crosslink DNA to the protein coat and then grow poly(dT) tails. This procedure has the additional advantage that the DNA network stabilizes the protein coat, probably because of multipoint attachment, and thus decreases the tendency of the gold sol to coagulate. Furthermore, this negatively charged molecular network greatly decreases the nonspecific sticking of the gold spheres to chromosomes as well as to other surfaces.

Poly(dA) and poly(dT) hybridize rapidly with each other at room temperature at moderate electrolyte concentrations. Therefore, the indirect labeling method avoids exposure of the gold spheres to the more stringent hybridization conditions needed for hybridizing an irregular cloned sequence to its chromosomal site.

We wish to note one technical point. dTTP is a better substrate than dATP for the enzyme terminal transferase. For this

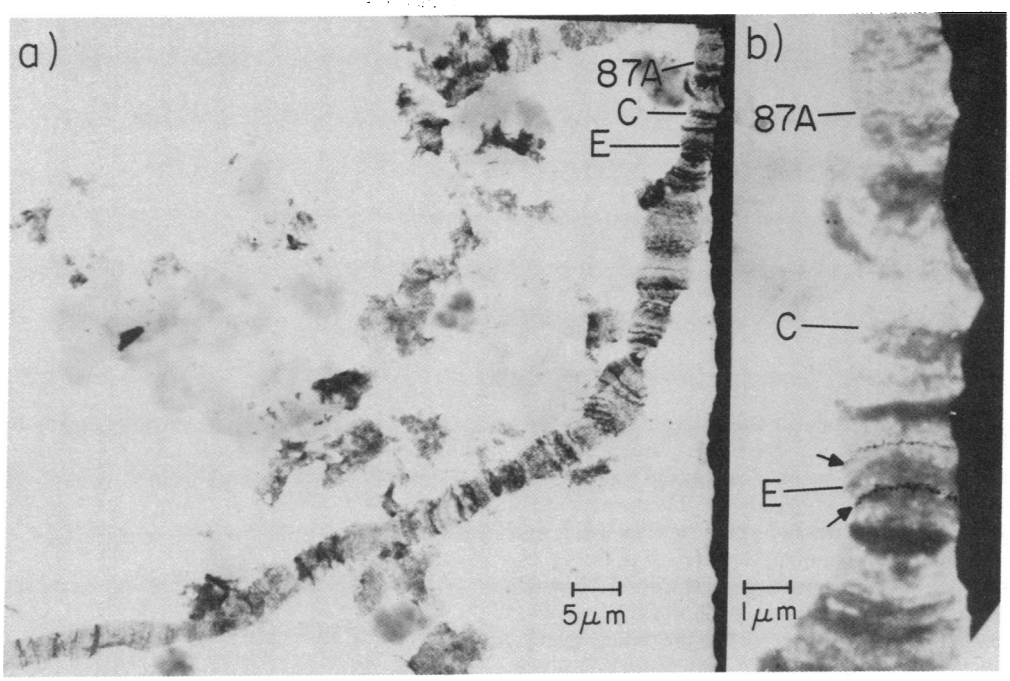

FIG. 5. (*a*) Transmission electron microscope image of chromosome 3X hybridized with cDm2848–poly(dA)/cDm2188–poly(dA)/gold-dT at low (×600) magnification. (*b*) Part of the same chromosome at a higher (×7100) magnification shows two bands of gold spheres (arrows) at the 87D and E regions.

reason, we chose to add the poly(dT) tails to the gold spheres first, because they are a more complex and less well-defined system, and then add the poly(dA) tails to the nicked cloned DNA.

As noted above, we have not yet succeeded in counting the number of hybridized spheres. Further studies are needed to determine the sensitivity of the method and whether, for example, it can be applied to mapping single-copy sequences in mammalian metaphase chromosomes.

It is noteworthy that chromosomes, when suitably cleaned and properly extended by squashing, are sufficiently thin so that they are suitable for examination by transmission electron microscopy at 60–80 keV.

The linear density of base pairs (in units, for example, of kb per μm) on the polytene chromosomes varies in different squashes, depending on the degree of stretching of the chromosomes; it may depend also on the degree of DNA condensation in the different bands. We observe apparent densities of ≈60 kb/μm for the cluster of 5S genes in the typical squash shown in Fig. 4*b* and of 293 kb/μm for the region 87D11 to 87E5 in the squash of Fig. 5*b*. The above mentioned factors and other unknown structural factors may contribute to these different observed linear densities.

Our subjective judgment from comparison with published photographs is that the present method provides much higher resolution than conventional *in situ* hybridization using ^{3}H- or ^{125}I-labeled molecules and autoradiography. The micrograph of the two single-copy cloned sequences that map at regions 87 D11 and 87 E5 shows two very sharp bands of hybridization. The bands are known to be 266 kb apart. We judge that bands separated by about one-fifth the separation of the two bands in Fig. 5 would be fully resolved. Further studies with cloned sequences are necessary to evaluate the practical resolution of this method. It may also be possible, with the resolution now available, to make significant observations on the relative positions of structural genes and other sequences relative to the bands and interbands seen in polytene chromosomes.

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